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Cell diameter measurements obtained with a handheld cell counter could be used as a surrogate marker of G2/M arrest and apoptosis in colon cancer cell lines exposed to SN-38

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ABSTRACT

In vitro assessment of chemosensitivity are important for experiments evaluating cancer therapies. The Scepter 2.0 cell counter, an automated handheld device based on the Coulter principle of impedance-based particle detection, enables the accurate discrimination of cell populations according to cell size and volume. In this study, the effects of SN-38, the active metabolite of irinotecan, on the colon cancer cell lines HCT116 and HT29 were evaluated using this device. The cell count data obtained with the Scepter counter were compared with those obtained with the ^{3}H-thymidine uptake assay, which has been used to measure cell proliferation in many previous studies. In addition, we examined whether the changes in the size distributions of these cells reflected alterations in the frequency of cell cycle arrest and/or apoptosis induced by SN-38 treatment. In our experiments using the Scepter 2.0 cell counter, the cell counts were demonstrated to be accurate and reproducible measure and alterations of cell diameter reflected G2/M cell cycle arrest and apoptosis. Our data show that easy-to-use cell counting tools can be utilized to evaluate the cell-killing effects of novel treatments on cancer cells *in vitro*.

1. Introduction

Various methods, such as the colony formation assay [1], the MTT (3-[4,5-dimethylthiazol-2-yl],5-diphenyltetrazoliumbromide) assay [2,3], the trypan blue assay [4], the BrdU (5-bromo-2′-deoxyuridine) incorporation assay [5] and the ^{3}H-thymidine incorporation assay [6,7], have been used to assess *in vitro* cell proliferation as a means of evaluating the treatment effects of chemotherapeutic agents, ionizing radiation or other cell-killing agents. The colony formation assay is considered to be the standard method for evaluating the effects of such treatments on cell proliferation *in vitro*, but it takes a relatively long time (about 2 weeks), and it is sometimes difficult to evaluate colony formation via microscopic observations. The MTT assay is a colorimetric assay that is used to determine the activity of cellular enzymes that reduce tetrazolium salts to yield insoluble formazan dyes, and the WST (water soluble tetrazolium salt) assay is a colorimetric assay that uses a water soluble tetrazolium salt and yields a water soluble formazan dye [8]. These colorimetric assays measure the cellular metabolic activity of NAD(P)H-dependent oxidoreductase enzymes, so their results indirectly reflect cell viability. These assays are relatively simple and quick, however, their results can sometimes be misleading. For example, it has been reported that soluble formazan assays can be affected by the medium used, serum albumin and fatty acids [9]. In the ^{3}H-thymidine incorporation assay, the radioactivity of DNA-incorporated ^{3}H-thymidine is measured with a scintillation beta-counter. This assay is an accurate indicator of DNA synthesis, but has to be performed in a laboratory equipped with a microplate scintillation counter.

Direct cell counting using a microscope and hemocytometer is laborious and time-consuming and so cannot be used as a routine examination method for evaluating the cell-killing effect of a novel compound. Various automated cell counters have been developed as bench-top systems based on the principles of conventional

Abbreviations: MTT, 3-[4,5- dimethylthiazol-2-yl],5-diphenyltetrazoliumbromide; BrdU, 5-bromo-2′-deoxyuridine; WST, water soluble tetrazolium salt; NAD(P)H, nicotinamide adenine dinucleotide (phosphate); PI, propidium iodide; CV, coefficients of variation; MSI, microsatellite instability.

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image analyzers and/or flow cytometry. The Merck Millipore Corporation have developed a handheld automated cell counting tool, the Scepter 2.0 cell counter, which uses the Coulter principle of impedance-based particle detection and displays reliable accuracy [10]. Furthermore, this system can also assess the size and volume of cells and obtains more information than other cell counting tools.

Irinotecan is a major chemotherapeutic drug that acts as an inhibitor of topoisomerase I, which is involved in breakage-reunion reactions during the DNA replication and translation of DNA [11]. Irinotecan is a prodrug that is converted to SN-38 by the action of carboxylesterase (EC 3.1.1.1) *in vivo* [12,13]. SN-38 is the active metabolite of irinotecan and induces double stranded DNA breaks, cell cycle arrest at G2/M and apoptosis in colon cancer cells [14,15].

Here, we evaluated the treatment effects of SN-38 on colon cancer cell lines using the Scepter 2.0 cell counter. The obtained data were compared with those derived from other assays such as the ^{3}H-thymidine incorporation assay; a cell cycle assay measuring the nuclear fluorescence intensity of propidium iodide (PI); and apoptosis assays using annexin V, calcein AM and PI staining. The aim of this study was to examine the correlation between cell diameter and nuclear DNA content and/or apoptosis and to validate whether the Scepter 2.0 cell counter can be used to assess the effect of a novel compound *in vitro*.

2. Materials and methods

2.1. Cell lines and culturing

The human colon cancer cell lines HCT116 and HT29 were purchased from the American Type Culture Collection (ATCC; Summit Pharmaceutical International, Tokyo, Japan). The cells were cultured in RPMI1640 medium (Life Technologies Japan, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific Japan, Yokohama, Japan) and 100 μg/ml kanamycin (Sigma–Aldrich, St. Louis, Mo, USA) at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Drug

SN-38 (Sigma–Aldrich) stock solutions were prepared by dissolving SN-38 in dimethyl sulfoxide (DMSO) to a concentration of 10 mM, and stored at −20 °C.

2.3. Cell count assay using the Scepter 2.0 cell counter

Cells were trypsinized and harvested during the exponential growth phase, and a total of 1–3 × 10^4 cells were plated in 24-well culture plates (500 μl/well). After 24 h, the cells were exposed to various concentrations of SN-38 for the indicated time periods. In the cell counting experiments using the Scepter 2.0 cell counter (Merck Millipore Japan, Tokyo, Japan), the cells were trypsinized and resuspended in culture medium. After the resultant cell suspensions had been transferred to 1.5 ml microcentrifuge tubes, the cells were counted with a Scepter 2.0 cell counter equipped with a 60 μm sensor tip, according to the manufacturer's recommendation. Cell counts and cell size distributions were shown as histograms on the monitor of the Scepter 2.0 cell counter, and these data were analyzed with the Scepter 2.0 Software Pro computer software. Before the cells were counted, the upper and lower gates of the counter were adjusted manually to eliminate small particles. Data regarding cell diameter are presented as the mean ± standard deviation (SD) values of triplicated experiments.

2.4. ^{3}H-thymidine incorporation assay

A total of 2–4 × 10^3 cells in 100 μl of culture medium were plated in 96-well culture plates. After 24 h, the cells were exposed to various concentrations of SN-38 for 48 h. ^{3}H-thymidine (1 μCi/well) was added to each well, and the plates were incubated at 37 °C for 2 h. Then, the ^{3}H-thymidine containing medium was discarded, and each well was washed twice with 100 μl of PBS. For cell fixation, 100 μl of 5% trichloroacetic acid (TCA) were added to each well, and then the plates were incubated on ice for 10 min. After discarding the 5% TCA, 50 μl of 1 N NaOH were added to each well, and the plates were incubated at 37 °C for 10 min. Next, 30 μl of the cell lysate were transferred to 96-well LumaPlates (Perkin-Elmer, CA, USA) and air-dried. Finally, the radioactivity of ^{3}H-thymidine was assessed using the TopCount NXT microplate scintillation counter (Perkin-Elmer). Data are expressed as mean ± SD values for replicated wells (n = 6).

2.5. Analysis of DNA content using the IN Cell Analyzer 1000

The DNA contents of the cell nuclei were measured by PI staining using the IN Cell Analyzer 1000 (GE Healthcare Japan, Tokyo, Japan). A total of 2–4 × 10^3 cells in 100 μl of culture medium were plated in 96-well culture plates. After 24 h, the cells were exposed to various concentrations of SN-38 for 48 h, before being washed twice with 100 μl of PBS. For cell fixation, 100 μl of 70% ethanol were added to each well, and the plates were incubated at 4 °C for 60 min. The ethanol was then discarded, and 100 μl of PBS containing 0.2% Triton X-100, 1 μl of PI (BioVision, PA, USA), and 1 μl of RNase A (Worthington Biochemical Co., Lakewood, USA) were added to each well, before the plates were incubated at room temperature for 60 min. The PBS containing Triton X-100, PI, and RNaseA was discarded, and 100 μl of PBS were added to each well. The IN Cell Analyzer 1000 scanned images of 9 fields from each well, and the intensity of the fluorescence produced by PI was calculated with the IN Cell Investigator software (GE Healthcare Japan).

2.6. Apoptosis assay using fluorescence microscopy

Cell apoptosis was evaluated using the Annexin V-Biotin Apoptosis Detection Kit (BioVision). A total of 5 × 10^5 cells were resuspended in 200 μl of the binding buffer together with 2 μl of annexin V-biotin and 2 μl of PI, before being incubated at room temperature for 5 min. The cell suspensions were centrifuged, and the resultant pellets were resuspended in 200 μl of the binding buffer supplemented with 0.1 μl of calcein AM stock solution (1 mM) (Life Technologies Japan) and 0.2 μl of Cy5-labeled streptavidin (GE Healthcare Japan), before being incubated at room temperature in the dark for 10 min. The cell suspensions were centrifuged, and the resultant cell pellets were resuspended in 100 μl of PBS. The cell suspensions were then dropped onto slides and covered with cover slips. Microphotographs were acquired using a BZ-9000 fluorescent microscope (KEYENCE, Osaka, Japan).

2.7. Statistical analysis

Data are expressed as mean ± SD values. The significance of differences in cell diameter, SN-38 exposure time, and/or SN-38 concentration was analyzed by two-way repeated measures ANOVA and the square of the correlation coefficient (r^2) was analyzed using Pearson's correlation test to assess the correlation between the Scepter cell count and ^{3}H-thymidine uptake or between the percentage of cells in the high diameter region and the percentage of tetraploid (4n) cells. All statistics were calculated using the GraphPad Prism V5.0 software (GraphPad Software, Inc., San Diego,